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ABSTRACT

A nucleic acid-based multiplexed assay was developed that combines detection of foot-and-mouth disease virus (FMDV) with rule-out assays for two other foreign animal diseases and four domestic animal diseases that cause vesicular or ulcerative lesions indistinguishable from FMDV infection in cattle, sheep and swine. The FMDV "look-alike" diagnostic assay panel contains five PCR and twelve reverse transcriptase PCR (RT-PCR) signatures for a total of seventeen simultaneous PCR amplifications for seven diseases plus incorporating four internal assay controls. It was developed and optimized to amplify both DNA and RNA viruses simultaneously in a single tube and employs Luminex™ liquid array technology. Assay development including selection of appropriate controls, a comparison of signature performance in single and multiplex testing against target nucleic acids, as well of limits of detection for each of the individual signatures is presented. While this assay is a prototype and by no means a comprehensive test for FMDV "look-alike" viruses, an assay of this type is envisioned to have benefit to a laboratory network in routine surveillance and possibly for post-outbreak proof of freedom from foot-and-mouth disease.

Key Words: Multiplex RT-PCR FMDV Rule-out

1. INTRODUCTION:

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting domestic and wild ruminants and swine, and is characterized by vesicles on the tongue, gums, nose and feet. FMD has not been seen in the United States since 1929 but the disease is endemic in many parts of the Middle East, Africa, Asia and South America. Periodically there are outbreaks in countries previously free of the disease, as demonstrated by the outbreak of FMD in the UK in 2001 (Haydon et al. 2004). That outbreak required the destruction of millions of animals, and the disposal of their remains (Scudmore et al. 2002, Rweyemamu and Astudillo 2002). Travel and trade restrictions had an estimated economic impact of more than $12 billion on travel, tourism, and many agriculturally-related industries (Thompson et al. 2002).

Diagnosis of FMD is confounded by other vesicular diseases that induce clinical signs in animals that are similar to those produced by FMDV. Testing for FMD and "look-alike" diseases with conventional real-time RT-PCR requires performing multiple individual disease-specific assays. Combining individual assays for FMDV and "look-alike" diseases into a single diagnostic assay capable of differentiating FMDV from "look-alikes" could provide a very rapid, cost-effective means of viral identification. Additionally, such an assay, if used for routine testing of diagnostic samples would significantly expand the number of tests performed on each sample, without significantly increasing the costs of the overall tests, or the time to results. This type of testing enables routine surveillance for foreign animal diseases that might otherwise go undetected, and affords early detection, maximizing the amount of time available to the United States Department of Agriculture/Animal and Plant Health Inspection Service [USDA/APHIS] to respond to a foreign animal disease outbreak.

A multiplexed reverse transcriptase polymerase chain reaction (RT-PCR) liquid array assay was developed for the simultaneous detection and differentiation of FMD virus from six other viruses that cause vesicular diseases. The liquid array technology employed for this assay is described in the methods section. Selection of viruses for inclusion in the panel was conducted in collaboration with the Department of Homeland Security (DHS) and the USDA/APHIS and based on end-user requirements. The virus panel combines liquid array-based testing for the detection of *Foot and mouth disease virus* (FMDV) [Family: *Picornaviridae* Genus: *Aphthovirus* Species: *Foot-and-mouth disease virus*] two additional foreign animal diseases including *Swine vesicular disease virus* (SVDV) [Family: *Picornaviridae*, Genus: *Enterovirus*, Species: *Porcine enterovirus B*, Serotype: *Swine vesicular disease virus*] and *Vesicular exanthema of swine virus* (VESV) [*Fmily: Caliciviridae*, Genus: *Vesivirus,* Species: *Vesicular exanthema of swine virus*] and four domestic "look-alike" disease viruses, including *Bovine herpes virus-1* (BHV-1 aka infectious bovine rhinotracheitus) [Family: *Herpesviridae*, Subfamily: *Alphaherpesvirinae*, Genus: *Varicellovirus,* Species: *Bovine herpesvirus 1*] parapox viruses including *Bovine papular stomatitis virus* (BPSV), *Pseudocowpox virus* (PCPV) and *Contagious ecthyma of sheep virus* (Orf virus) [Family: *Poxviridae,* Subfamily: *Chordopoxvirinae*, Genus: *Parapoxvirus,* Species: *Bovine papular stomatitis virus*, *Pseudocowpox virus*, and *Orf virus*], plus *Bovine viral diarrhea virus* (BVDV) [Family: *Flaviviridae,* Genus: *Pestivirus*, Species: *Bovine viral diarrhea virus 1*], and domestic *Bluetongue virus* (BTV) [Family: *Reoviridae*, Genus: *Orbivirus*, Species: *Bluetongue virus*].

The multiplexed assay panel (Table 1) comprises seventeen virus detection signatures, each consisting of a forward primer, reverse primer and probe designed to target unique genomic sequences of specific viruses. The two signatures for detection of FMD used in this multiplex

assay were developed by others and have been previously used for FMD laboratory diagnosis. One signature (5´UTR Pirbright) targets the ribosomal entry site of the 5´untranslated region (Reid et. al 2002) while the other signature (3D Tetracore) targets the viral RNA polymerase gene (Callahan et. al. 2002) on the FMDV genome. All viruses in the panel, with the exception of BVDV, are represented by two or more signatures. Additional signatures for the detection of BVDV that can be incorporated into the multiplexed panel are under development. Employing multiple signatures for a pathogen within the same assay, where those signatures target different genomic regions of the pathogen, increases the specificity of an assay and reduces the risk of false positives. Using multiple signatures is even more important in an assay designed to detect rapidly-mutating pathogens like FMD where gene targets may change between virus generations allowing for the pathogen to escape detection. Such a gene target deletion has recently been identified for the bacteria *Chlamydia trachomatis* in a 377bp site in a plasmid used for clinical diagnosis of this pathogen by PCR (Ripa and Peter 2007). Our detection algorithms require that all signatures for a pathogen must be positive in order for the sample to be ruled positive. The assay also includes four internal controls. The controls provide significant levels of increased confidence in the multiplexed assay results, as they have been designed to monitor and report each step of the assay. The development, optimization and evaluation of the assay is described, a comparison of assay performance in both the singleplex and multiplexed environments is presented, and the utility of the assays for the simultaneous detection of multiple viruses from a single sample is demonstrated.

2. MATERIALS AND METHODS

2.1Liquid Bead Based Multiplex Array The multiplexed assay was developed using Luminex™ flow cytometry-based technology. The liquid arrays (Figure 1a) utilize surface-functionalized polystyrene microbeads embedded with unique ratios of two fluorophores (red and infrared). Each unique dye ratio results in a distinct emission profile, a unique "spectral address". Because the microbead classes can be distinguished, they can be combined so that up to 100 different analytes can be measured simultaneously within the same sample. The versatility of the liquid arrays has been well-demonstrated for detection of antibodies, antigens, and peptides (McBride et al. 2003, Dunbar et al. 2003, Kellar and Iannone 2002, Perkins et al. 2006). For nucleic acid-based detection, oligonucleotide probes with sequences that are complimentary to target nucleic acid sequences are covalently coupled to individual bead sets (Figure 1b). These individual bead sets are then formulated into a mixture in a 1:1 ratio of all bead types. Nucleic acids from pathogens (targets) are amplified using standard PCR techniques (Figure 1c). After target amplification, the amplicons, half of which contain the biotinylated forward (5'-3') primer, are introduced to the bead mixture, and allowed to hybridize to their complimentary probes on the corresponding beads (Figure 1d). A fluorescent reporter molecule, strepavidin-phycoerythrin (SA-PE), is added and binds the biotin functional groups within the forward primers (Figure 1e). The completed assay product is comprised of a bead + probe + biotinylated and fluorescently tagged amplicon (Figure 1e). Each optically encoded and fluorescently labeled microbead is then analyzed by the flow cytometer. Each individual bead within the sample is rapidly analyzed: the 635-nm laser excites the dyes inside the bead, and classifies each bead to its unique bead class, while a 532-nm laser quantifies the assay at the bead surface. Assays can be conducted from sample prep to results in a 96-well format in about three hours.

2.2Buffers and Reagents Tris-NaCl (0.1 M Tris, 0.2 M NaCl, 0.05 % Triton X, pH = 8.0), MES (0.1M 2-{N-morpholino}ethanesulfonic acid, 0.02 % Tween-20, 0.1 % SDS (sodium dodecyl sulfate), pH = 4.5), TE (Tris-EDTA, 10 mM Tris-HCl, 1.0 mM EDTA, pH = 8.0) buffers were purchased as custom formulations from Teknova Inc. (Hollister, CA). Streptavidin-R-phycoerythrin (Caltag Laboratories/ Invitrogen, Carlsbad, CA) was prepared and stored as a 100X concentration (300 µg/mL) in Tris-NaCl, and diluted to a working concentration of 3.0 µg/mL just prior to use.

2.3 Viruses The following titered stocks of virus were purchased from the National Veterinary Service Laboratory(NVSL) (Ames, Iowa): BHV type 1(Colorado vaccine strain) in embryonic bovine kidney (EBK) cells, BTV serotypes 2 and 13 (no strain given) in baby hamster kidney (BHK) cells; BVDV genotype 1 (Singer strain, cytopathic) in EBK cells, and BPSV (Texas A&M strain) in EBK cells. The following seed viruses were purchased from the NVSL, grown and titered in BHK cells at LLNL: BTV-10 (lot #001 ODV 0001 Dec. 5, 2000, no strain given); BTV-11 (lot #002 ODV 0101 Nov. 30, 2001, no strain given); BTV-17 (lot #004 ODV 0201 Nov. 28, 2002, no strain given). The following viruses were also grown and titered at LLNL: BHV-1 (L.A. strain) and BHV-1 (Texas strain) in Madin-Darby bovine kidney (MDBK) cells; *Ovine contagious ecthyma* (orf) vaccine virus (commercial strain) in embryonic bovine lung (EBL) cells; and BVDV (Singer strain) in MDBK cells.

The following foreign animal disease viruses (see supplemental data for strains used) were grown at Plum Island Animal Disease Center (PIADC): *Swine vesicular disease virus* (SVDV) in SK6 swine kidney cells, *Vesicular exanthema of swine virus* (VESV) in Vero cells, and *Foot-and-mouth disease virus* (FMDV) in BHK cells. The viruses were inoculated into flasks of 80% confluent cells and incubated at 37°C in 5% CO_2. The BHK, EBK, Vero and SK6

cells were grown in Eagle's minimal essential medium with Earle's salts and nonessential amino acids. Maintenance media also contained 2-4% fetal bovine serum, 2mM L-glutamine, 1.0 mM sodium pyruvate and added antibiotics. When cytopathic effect (CPE) reached 75-100%, the flasks were frozen at -70°C. The contents were later thawed, clarified and the supernatant aliquotted and stored at -70°C. $TCID_{50}$ titrations were performed in quadruplicate in 96 well plates. $TCID_{50}$ values were calculated by the Spearman-Karber method (Finny 1978) for those viruses grown at NVSL and Plum Island and by the method of Reed and Muench (Reed and Muench 1936) for those grown at LLNL.

2.4 Target Nucleic acids. Ninety one target virus strains and 51 near-neighbor virus strains were provided by collaborating diagnostic and reference laboratories and used to develop, optimize, and characterize the assays. Near-neighbor strains are defined as genetically closely-related viruses.. Target viruses used for testing and optimization purposes are listed in Table 2, with additional details provided in the supplemental material. BVDV, (single-stranded positive sense) and BTV (double-stranded) RNAs were extracted with twice the volume of Trizol (Invitrogen, Carlsbad, CA), mixed, and incubated 15 min at 25°C. One-fifth of the total volume of chloroform was added, mixed, incubated 15 min at 25°C and centrifuged at 3000 x g 15 min at 4°C. To the aqueous layer was added one half volume of isopropyl alcohol. The sample was mixed, incubated 10 min at 25° C, and then centrifuged 10 min at 12,000 x g at 4°C. The pellet was washed with 70% ethanol, dried briefly at 55°C, dissolved in RNAse-free water and stored at -80 ° C. A measure of extraction efficiency was made using quantitative rT-PCR. The amount of RNA recovered from the direct extraction of a known amount of purified tobacco mosaic virus (TMV) was compared to the amount of TMV RNA recovered by extraction of the

same amount of virus from a culture supernatant containing titered BVDV or BTV. By this method, the Trizol RNA recovery efficiency was determined to be 98%.

BPSV, Pseudocowpox, orf and BHV-1 DNA was extracted using a modification of a standard phenol/chloroform/ isoamyl alcohol protocol. Triton X-100 (Sigma, St Louis, MO) and EDTA (Sigma) were added to virus samples to a final concentration of 0.5% and 20 mM, respectively. The mixture was vortexed, incubated 5 min. at 25 ° C, and centrifuged at 1000 x g for 10 minutes; 10% sodium dodecyl sulfate (SDS) solution (Sigma) and Proteinase K (Roche, Indianapolis, IN) were added to a final concentration of 1 % and 4U/ml, respectively. The sample was held one hour at 55°C with vortexing every 10 minutes, and allowed to cool to room temperature with the addition of NaCl to a final concentration of 150 mM. An equal volume of phenol/chloroform/isoamyl alcohol (Sigma) in a 25:24:1 ratio was added, and the solution mixed thoroughly. After 5 minutes at 25°C, the mixture was centrifuged at 3,000 x g for 10 minutes. The aqueous layer was aliquotted (500 µl per tube) into microfuge tubes. To each tube, 1 ml of 100% ethanol was added. The tubes were incubated one hour at -20°C and then centrifuged at 18,000 x g for 10 minutes at 4°C. The pellets were washed first with ice-cold 100% ethanol, next with ice-cold 70% ethanol containing 150mM NaCl, air-dried briefly, dissolved in 10 mM Tris-1 mM EDTA (TE) buffer and stored at -20°C less than one month. DNA extraction efficiency was determined by adding 50 pg of pUC18 to 0.95 ml of each virus sample before extraction. The extracted virus/pUC18 mixtures were serially diluted 1:10 and assayed by quantitative PCR (qPCR). The concentration at which fluorescence levels failed to cross threshold in a pUC18 qPCR assay was compared to a second set of qPCR reactions using known concentrations of pUC 18. The efficiency of extraction was determined to be 100%.

At Plum Island SVDV, VESV, FMDV were extracted using an Ambion Mag Max -96 total RNA isolation kit (Catalog #AM 1830) as per manufacturer's protocols.

2.5 Background Nucleic acids. Panels of "background" nucleic acid extracts were also used to determine specificity of the assays. These included total nucleic acid extracts from 50 soil samples taken from multiple urban, suburban, and rural sites within the United States and during multiple seasons, 54 total nucleic acid extracts from prokaryotes, and 13 total nucleic acid extracts from eukaryotes (see supplemental materials for complete list of prokaryote and eukaryote backgrounds used).

2.6 PCR Primers. Candidate signatures (primer-probe triplets designed to target specific regions of a genome) were identified and selected using "KPATH", a whole-genome nucleic-acid signature design system (Slezak et al. 2003). All available complete or partial genomes for the virus targets in the multiplexed panel were computationally examined to identify sequence regions that were conserved among all sequenced isolates for the pathogen of interest but also unique to the target pathogen when compared against all available genomic sequence data in the KPATH database. The conserved/unique sequence information was used to develop candidate signatures that met TaqMan and multiplex assay chemistry requirements.

Oligonucleotide primers at 90% or more purity as determined by HPLC, were purchased as lyophilized pellets from Integrated DNA Technologies (IDT DNA, Coralville, IA). Purification of each lot was assessed by mass spectrometry and capillary electrophoresis. A 100 μM solution of primers was prepared in 10 mM TE buffer. Forward primers were purchased with 5' biotin modifications and up to two additional internal biotin moieties. Since biotin molecules are larger than nucleotide bases, the biotin molecules were separated by five to ten bases and were not placed at nucleotides next to the 3' terminus of the forward primer to avoid interference with

amplification. Internal biotin placement in the forward primers depended on thymidine location and quantity since thymidine is the attachment site for internal biotins.

2.7 Probe Coupling to Luminex microbeads. Oligonucleotide probes were assigned to different sets of carboxylated fluorescent microbeads (Luminex Corp., Austin, TX). To enable optimum hybridization, each probe sequence represented the forward compliment to the target region of the reverse strand (3'-5'), and contained a C-18 spacer between the reactive groups on the beads and the 5' end of the oligonucleotide. Probes for each of the pathogen targets were coupled to the beads using the manufacturer's recommended coupling protocol (Wilson et al. 2005).

2.8 Preparation of Bead Mixture. Bead mixtures were formulated for a 100 reaction mix where the total volume of 1X bead mix needed is 2200ul. Four microliters of each conjugated bead class were mixed together with a volume of Tris-NaCl buffer sufficient to make the final volume of 2200ul. To ensure that each microbead class was present in the bead mixture in approximately equal amounts, a 22 µl aliquot was pulled from the 1X mixture, mixed with 78ul of Tris-Nacl (see buffers and reagents section) and analyzed with a Bio-Plex (Bio-Rad Laboratories, Hercules, CA) instrument. This ratio of 22:78 bead mixture to Tris-Nacl was used as it is equivalent to the concentration of beads in the final solution counted by the instrument after PCR hybridization (sec 2.10 below). The instrument was adjusted to count all bead classes in the sample to 1000 beads per bead class.. If the bead count of a particular class was low, a compensatory amount of that bead coupled to probe was added to the bead mixture. This was to insure that a particular microbead type is not limiting during the optical analysis. The bead mixtures were stored at 4 ºC in the dark before use.

2.9 PCR reagents. All reactions were performed in a total volume of 25 µl (20 µl master mix plus 5 µl sample) optimized for multiplexed rT-PCR. A volume of 20 µl rT-PCR master mix

(Superscript III One-Step rT-PCR System, Cat#12574-026 Invitrogen, Carlsbad, CA) contains: 12.5 µl 2X buffer (0.4 mM dNTPs, 3.2 mM $MgSO_4$, and stabilizers), 1 µl Platinum Taq DNA polymerase and reverse transcriptase, 0.95 µl 50 mM $MgSO_4$ (3.5 mM MgSO4 final concentration), 3.6 µl primer mix (0.4 µM each forward and reverse primer final concentration) 1.0 µl Alien- armoredRNA @ 100copies/µl (used as internal control) and 0.95 µl water (Teknova). The armored RNA (XenoRNA-01, Ambion, Austin, TX) is a proprietary 1070 nucleotide RNA transcript consisting of unique nucleotide sequences that possess no significant homology to the current annotated sequences in commonly used sequence databases.

2.10 Thermal cycling conditions: Reverse transcription for cDNA synthesis was carried out at 55º C for 30 minutes, followed by inactivation of the reverse transcriptase, activation of Platinum Taq DNA polymerase, and denaturation of the RNA/cDNA hybrid at 95°C for 2 minutes. Amplification was performed in a 96-well MJ Tetrad thermocycler (Bio-Rad) for 35 cycles at 95ºC for 15 s, 60ºC for 30 s, 72ºC for 15 s, followed by a final extension of 72°C for 2 minutes and a 4°C hold.

2.11 Hybridization reaction: Following rT-PCR amplification, 5 µl of PCR product was added to 22 µl of microbead mixture. The amplified product was denatured at 95°C for 2 minutes, and hybridized at 55°C for 5 minutes, and a 4°C hold. Following hybridization 100 µl of Tris-NaCl buffer was added to the beads and this mixture was transferred to a pre-wetted 96-well filter bottom plate, 1.2 µm pore size (Millipore Corp., Billerica, MA), and washed 3x with 100 µl Tris-NaCl to remove nonhybridized nucleic acids. The PCR products bound to the beads were labeled by adding 60 µl SA-PE (streptaviden R-phycoerythrin conjugate 3 ng/µl) and were incubated in the dark for 5 minutes. Beads were washed once with Tris-NaCl, re-suspended in 100 µl Tris-NaCl and analyzed in a Bio-Plex.

2.12 Titrations to Determine Limits of Detection: Serial dilutions of titered nucleic acids (as described above) spanning eight logs of concentrations were prepared and tested to assess the sensitivity and specificity of multiplexed assays.

3. RESULTS AND DISCUSSION

3.1 Assay Development, Optimization, and Characterization. Primers from the KPATH design process (see methods 2.6) were tested against an extensive "background" panel of nucleic acids derived from potentially-cross-reacting, interfering, or confounding sources to ensure that the primers detected the strain diversity of each pathogen but did not react with other nucleic acids that could be present in a sample. Primer pairs that successfully passed this wet-chemistry screening criteria were advanced to assay development, where extraction, PCR thermocycling and reaction procedure were optimized for consistent performance. One by one, each primer pair was evaluated against the full compliment of probes. This approach allowed for the simultaneous assessment of individual assay sensitivity as well as specificity (as determined by the lack of reactivity of target nucleic acids with probes). Suitable primer-pairs were then systematically incorporated into the multiplexed panel. Using the complete panel of multiplexed signatures (Table 1), the screening was repeated with the backgrounds panel. The performance of each signature was characterized in both simplex (a single primer-probe pair assay) and multiplexed (more than one primer-probe pair in an assay) formats.

The data presented in Table 2 summarizes the results of each step of initial assay development, and underscores the stringency of this process. For example, KPATH identified 177 potential candidate signatures for BHV-1. Each signature passed tests for uniqueness and conservation, and was predicted to detect target sequences, but not closely related sequences, in a

simulated multiplexed assay. Of the 177 *in silico* candidate signatures, seventy-six were eliminated in the initial wet chemistry screening. Of the 101 remaining signatures, 97 were eliminated because they either did not detect all the target strains tested or they incorrectly cross-reacted with near-neighbor strains (those viruses that are closely related at the genetic level and therefore have the greatest likelihood of cross-reaction). Only four signatures were suitable for inclusion in a multiplexed panel. In further testing, only two of the four signatures performed well in the multiplexed environment. These two signatures were tested in a multiplexed format against thirteen different target strains to establish sensitivity, and twenty near-neighbors to determine specificity. Multiplex testing with targets involved titrations over eight logs of target dilution while single plex tests involved testing at one concentration of target to determine that a signature was reactive against a range of target strains. Thus as shown in Table 2 fewer target strains were screened in multiplex than in single plex but the same signature was used in both tests.

3.2 Internal Controls, Data Analysis and Interpretation. When the flow cytometer completes a measurement, two important data parameters are displayed for each bead class used in the assay: the median fluorescent intensity (MFI) and the total number of beads counted. Because the assays are conducted using a multi-step protocol and were designed for use in a large number of laboratories by many experimental scientists, a unique set of four rationally designed controls was developed (Table 1) and built into every sample to monitor and report on certain key steps of the assay. A bead-conjugated Maritima (MT-7) oligonucleotide serves as the negative control (NC). MT-7 is a conserved DNA sequence from a deep sea thermal vent microbe that does not match any published genomes of terrestrial organisms. It serves as a measure of nonspecific

binding in the multiplexed assay. In the absence of nonspecific binding, the MFI values for the NC MT-7 bead should remain consistently low. A biotinylated MT-7 (b-MT7) oligonucleotide sequence, conjugated to a different bead, serves as the fluorescence control (FC). The biotin moiety has a high affinity for avidin in the streptavidin phycoerythrin conjugate and confirms that the fluorescent labeling step of the assay was performed correctly. MFI values of the b-MT7 bead should be consistently high and constant from assay to assay. Instrument control (IC) is accomplished with a bead conjugated to a Cy3-labeled MT-7 (MT7/Cy3). Both Cy3 and SA-PE have similar fluorescent excitation and emission wavelengths. MFI values for the MT7/Cy3 beads confirm that the flow cytometer reporter optics are functioning correctly. Large fluctuations in the MFI values for MT7/Cy3 from sample to sample may indicate failure of the reporter laser. Alien armored RNA, a synthetic RNA sequence approximately 1,000 nucleotides in length, packaged in an MS2 phage capsid, serves as an internal control for nucleic acid purification, rT-PCR amplification, microbead array hybridization and detection (Hietala and Crossley 2006). These controls convey important diagnostic information regarding assay integrity; including reagent addition, quality and concentration; assay operator performance; and instrument stability. These controls do not compromise or limit assay capabilities in any way.

The raw data outputs from the flow cytometer typically require substantial analysis and interpretation, as compared to data outputs from conventional real-time PCR assays. To simplify this process and ensure uniform interpretation of sample results between laboratories, detection algorithms to guide interpretation were developed. Threshold MFI values for each signature were established that enable results to be classified as either positive (analyte present) or negative (analyte absent). Threshold determination for these assays and the mathematical principles that underlie our algorithm development will be described in detail in a manuscript in

preparation. A positive / negative designation was made for each unknown sample by comparing each individual signature's MFI value with a threshold. Threshold values for each signature were calculated based on the distribution of MFI responses to a large number (> 1000) of known negative clinical samples obtained from thirteen state veterinary diagnostic labs from throughout the United States. A threshold value for each individual signature was chosen such that the MFI values generated by each signature in response to the set of negative samples would exceed threshold at a rate of 0.005 (or 1 per 500 samples). To run the algorithm, MFI values for the four internal controls were checked first. If the MFI value for any control was out of range, assay results were deemed invalid. However, if the MFI for the Alien armored RNA control *and* one or more MFI values for the seventeen agents exceeded threshold, that sample were then deemed valid and included in further analysis. Agent MFI spikes above certain concentrations have been observed by our laboratory to cause a decrease in the MFI of the Alien armored RNA, probably due to competition in the PCR reaction. If the MFI of Alien armored RNA dropped below threshold on a sample considered negative for all signatures, the sample was repeated. This control reduces the probability of false negatives. If the MFI values for all four controls fall within range, the bead counts are checked. Minimum bead counts of 40 beads per bead class must be attained for the result to be considered valid. If a viral signature sample did not reach the 40 bead minimum, the assay result was deemed invalid for that viral sample. If the bead counts for any of the viral signatures exceeded the minimum, they were considered valid.

In addition to multiple internal controls, multiple signatures for a single virus enhances confidence in the assay result. A single signature, designed to target a specific genomic region may not detect certain strains that do not have that exact sequence in their genomes. The use of multiple signatures, where each signature targets different genomic regions of the same pathogen

increases the probability of detection and increases the number of strains or serotypes that the assay can detect. Our detection algorithms are designed to minimize the probability of false positives. For example, three signatures specific for a given virus are required to exceed their individual threshold values before a sample can be ruled positive. Each signature in the multiplexed assay has been selected such that it has a probability of false positive rate of 0.002. Thus, the likelihood of a false positive becomes 0.002^3 (approximately1 false positive per 100 million samples). Depending on signature design, the relative responses to different signatures may convey additional information about strain types or serotypes.

3.3 Comparison of Assay Performance in Singleplex versus Multiplexed formats. Figure 2 shows representative titration curves comparing the performance of individual signatures for the detection of three viruses (BHV-1, parapox, and BTV) in both simplex (closed circles) and multiplexed (open circles) formats. Dose-response curves were generated using nucleic acids extracted from the viral stock solutions described previously, diluted over eight log units (X-axis) and plotted against the log of the median fluorescent intensity (MFI) as reported by the flow cytometer software (Y-axis). Each data point of each curve represents the average of three replicate samples. Background fluorescence measured using PCR reagents with no nucleic acid target is given by the first data point in each curve.

Like the examples shown in Figure 2, all seventeen individual virus detection signatures in the multiplexed assay produced a reproducible response above background over 3-4 logs of concentration. The limit of detection (LOD) was defined as the target nucleic acid concentration at which the MFI value is 2.5 times the standard deviation of the background. For the signature comparison shown in Figure 2, no differences between the limit of detection obtained in simplex and multiplexed formats were observed.

Limits of detection for every signature within the multiplexed assay panel are summarized in Table 3. Limits of detection were generally lower for RNA viruses than for DNA viruses in the multiplexed format. This may have been due to the fact that the total number of RNA viruses was much higher than the number of RNA virions. Evidence indicates large differences in the ratio of viral particles to plaque-forming units [PFU] or infectious units for different RNA viruses. Different strains of poliovirus which, like FMDV, is a member of the Picornaviridae family, have been shown to have particle-to-PFU ratios ranging from 30 to 1,000 whereas strains of Semliki Forest virus, a member of the Alphaviridae, has a particle-to-PFU ratios of 1-2 (Flint et. al., 2004). Thus, the large variations in sensitivity differences may be due to variations in the number of infectious virions present. Alternatively, the one-step RT-PCR kit used in the comparison may not be optimal for the amplification of double-stranded DNA. Even the least sensitive assay, however, could detect 500 infectious units while the most sensitive could detect three logs less than one infectious unit.

The background fluorescence for each signature-bearing bead class (Table 3) was slightly different due to the inherent differences of the dye ratios in the beads in addition to the non-specific interaction of their probe moiety. In general, the baseline MFI values were between 5-20 in the simplex format (data not shown), and 5-65 in the multiplexed format. Background fluorescence values tended to be higher in the multiplexed format, most likely due to the more complex chemical environment produced by the large number of primers and/or due to an increase in nonspecific binding.

3.4 Simultaneous Detection of Nucleic Acids using the Multiplexed Assay Panel

To determine if the multiplexed RT-PCR assay could simultaneously detect multiple nucleic acids from different viruses in a single tube, samples containing both RNA and DNA from

domestic look-alike viruses were tested. Identical concentrations of BHV-1, BPSV, BVDV, and BTV, were combined to produce a master stock. The results are shown in the three-dimensional plot (Figure 3). The plot displays the results of eight individual assays, where each sample represents a serial dilution spanning 7 logs (Z-axis) of the master stock. Assay results included all of the 17 signatures and the four controls (X-axis): the controls have been omitted for clarity but were within expected limits (data not shown). MFI values are plotted on the Y-axis. The MFI values for each of the corresponding signatures increased with increasing concentration of mixed target nucleic acid, even in the presence of high concentrations of multiple targets. The nine signatures corresponding to the non-target viruses did not react even at the highest concentrations of amplified nucleic acids. These results demonstrate the high detection specificity of each component of the multiplexed assay panel. Moreover, despite the complex chemistry of the multiplexed assay, the simultaneous detection and differentiation of four different RNA and DNA viruses occurred with no increase in limit of detection (loss of sensitivity) for any signature with any target. Non-target foreign animal disease virus assays (FMDV, SVD, VESV) do not exhibit any elevated signal in complex viral sample matrices, illustrating the sensitivity and specificity of the multiplex assays,

The ability of the multiplex assay to detect multiple strains of targets was tested by using 1 ng of total RNA extract from virus culture supernatants showing cytopathic effect [CPE]. Thirty one FMDV isolates were tested in this way, and both FMDV signatures were found to be reactive to 29 of the 31 strains. Only the Tetracore signature showed a positive signal for two of four SAT2 isolates tested. Similarly, all four VESV signatures were positive for two of eight VESV isolates tested, with the remainder demonstrating different combinations of signatures showing positive signal on different isolates (see supplementary material). The signature

targeting the putative VPg gene (VESV-4) was most robust and was able to detect all eight isolates. This result is most likely a result of the conservation of this gene. The most robust individual SVD signature, showing a positive signal for all 27 isolates, was the SVD-1 signature targeting the viral capsid gene, while the least robust signature, SVD-3 targeting the viral replicase was positive for only 6 of 27 isolates tested. This result was unexpected as conservation of the replicase gene would be expected over and above that of the viral coat protein however other factors such as amplicon length and probe hybridization can affect the ability of a signature to detect a target virus genome. SVD-2 was positive for 25 of 27 isolates tested. For all isolates of BVDV, BTV, parapox, and BHV-1 target tested, all signatures targeting those agents were positive with no cross-reactivity seen.

Finally, as indicated in Table 2 and supplementary data, this multiplex assay does not show cross-reactivity with any of a large number of near-neighbor viruses tested. None of the 17 target signatures showed any cross-reactivity when tested against 1ng of total RNA extract from viral culture supernatants showing CPE collected from eight porcine enterovirus strains, six bovine enterovirus strains or human enterovirus coxsackie B5. Only the BVD-1a signature showed elevated MFI signals when the multiplex assay was tested against four epizootic hemorrhagic disease virus strains, a genetic near-neighbor to BTV. Further investigation revealed that the tissue cultures used to propagate these BTV near-neighbor viruses was contaminated with BVDV-positive fetal bovine serum. The elevated MFI signals for the BVD-1a were encountered with four equine herpesvirus and one feline herpesvirus isolates (all near-neighbors of BHV-1). Of all near-neighbor viruses tested, only the VESV-4 signature, the one demonstrated to be able to detect all VESV isolates, cross-reacted with the San Miguel sealion virus Type 2 [SMSV-2]. This result would be expected due to the close genetic relationship

between VESV/SMSV which shows these viruses belong to a single genotype distinct from other caliciviruses (Reid et. al. 2006).

In summary, the assay demonstrates robust detection of all targets tested with at least one signature showing positive signal for some, while multiple signatures showed positive signal on the vast majority of targets tested with no cross-reactivity observed. Additionally, no near-neighbor viruses showed any cross-reactivity with any of our signatures.

4. Conclusions

A first generation, rapid, highly multiplexed nucleic acid assay for the detection of FMDV and its differentiation from other viruses that cause symptoms indistinguishable from those of FMDV has been developed. Using seventeen virus detection signatures, the assay sensitivity in singleplex and multiplexed assay formats for several signatures has been shown to be comparable. A much more extensive evaluation of the overall assay sensitivity and specificity is underway in addition to test utilizing actual clinical samples. To demonstrate the robustness of the assays, an inter-laboratory comparison has been conducted at thirteen National Animal Health Laboratory Network (NAHLN) labs throughout the United States and at Plum Island Animal Disease Center (PIADC; results will be reported elsewhere).

The multiplexed liquid array-based assay described can be used for the simultaneous detection and differentiation of multiple DNA and RNA viruses in a single sample with the ultimate benefit of savings in both time and money, compared to traditional single tests for infectious agents. In the event of a disease outbreak, this assay approach can also be adapted to high throughput processing using robotic sample handling, sample barcoding and new data reporting software. This capability was demonstrated at the University of California, Davis in

May, and at Colorado State University in July of 2006. (results will be reported elsewhere). The assay described is a prototype and is by no means a comprehensive test for FMD "look-alike" viruses of cattle, pigs and sheep. Refinements to the prototype assay are currently underway, which include the development of two species-specific panels for cattle and pigs that provide more comprehensive FMDV "look-alike" virus coverage. These second-generation multiplexed assays will be more comprehensive, with a species-specific emphasis, and when fully-validated, could enhance preparedness and response capabilities of both state and federal veterinary diagnostics laboratories by enabling them to pair surveillance for FMDV with differential testing for the "look-alike" disease viruses.

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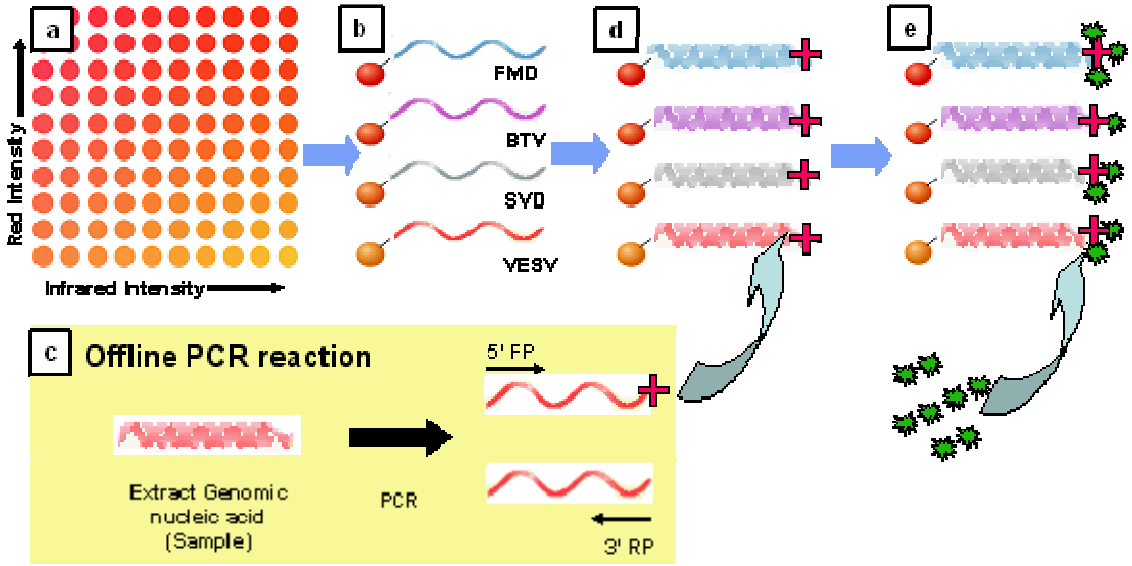

Figure 1. **A)** 100-plex liquid array generated by varying the ratios of red and infrared dyes embedded in polystyrene latex microbeads. Each optically encoded bead has a unique spectral address. **B)** Capture beads with covalently-coupled oligonucleotide probes complimentary to target nucleic acid. **C)** Individual primer pairs (biotinylated forward and standard reverse) that bracket the target genomic sequence are included in a PCR master mix of buffers, Taq polymerase, dNTPs, etc. After amplification by PCR, the amplicons are mixed with beads where target amplicons containing the forward biotinylated primers hybridize to the complimentary probe on the appropriate beads. **D)** A fluorescent reporter molecule (streptavidin-phycoerythrin) then binds biotin functional groups. **E)** The completed assay comprises a bead + probe + biotinylated (and fluorescently tagged) amplicon. The sample is then analyzed using the flow cytometer.

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Figure 2. Titration curves Singleplex/Multiplex

BHV-1 signature 3, BPSV signature 1, BTV signature 2

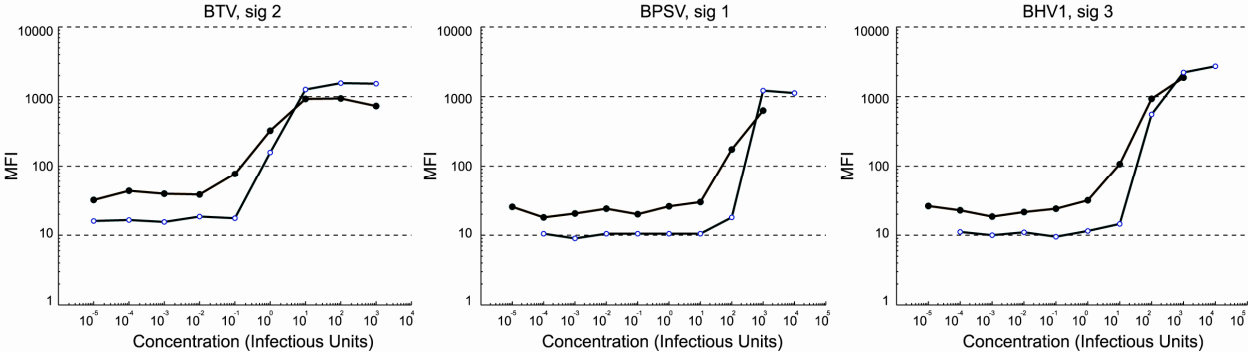

Figure 2. Log of the MFI vs virus RNA concentration in infectious units for a simplex (open circles) or multiplex (solid circles) assay. In each of the three plots, a signature is titrated against eight logs of diluted RNA or DNA from known amounts ($TCID_{50}$) of the target virus is shown. Left plot response of bluetongue virus signature 2 titrated against RNA extracted from BTV serotype 13. Center plot is the bovine papular stomatitis (BPSV) signature 1 titrated against DNA extracted from BPSV Texas A&M strain. The right plot is the response of bovine herpes virus (BHV-1) signature 3 titrated against DNA extracted from BHV-1 Colorado vaccine strain.

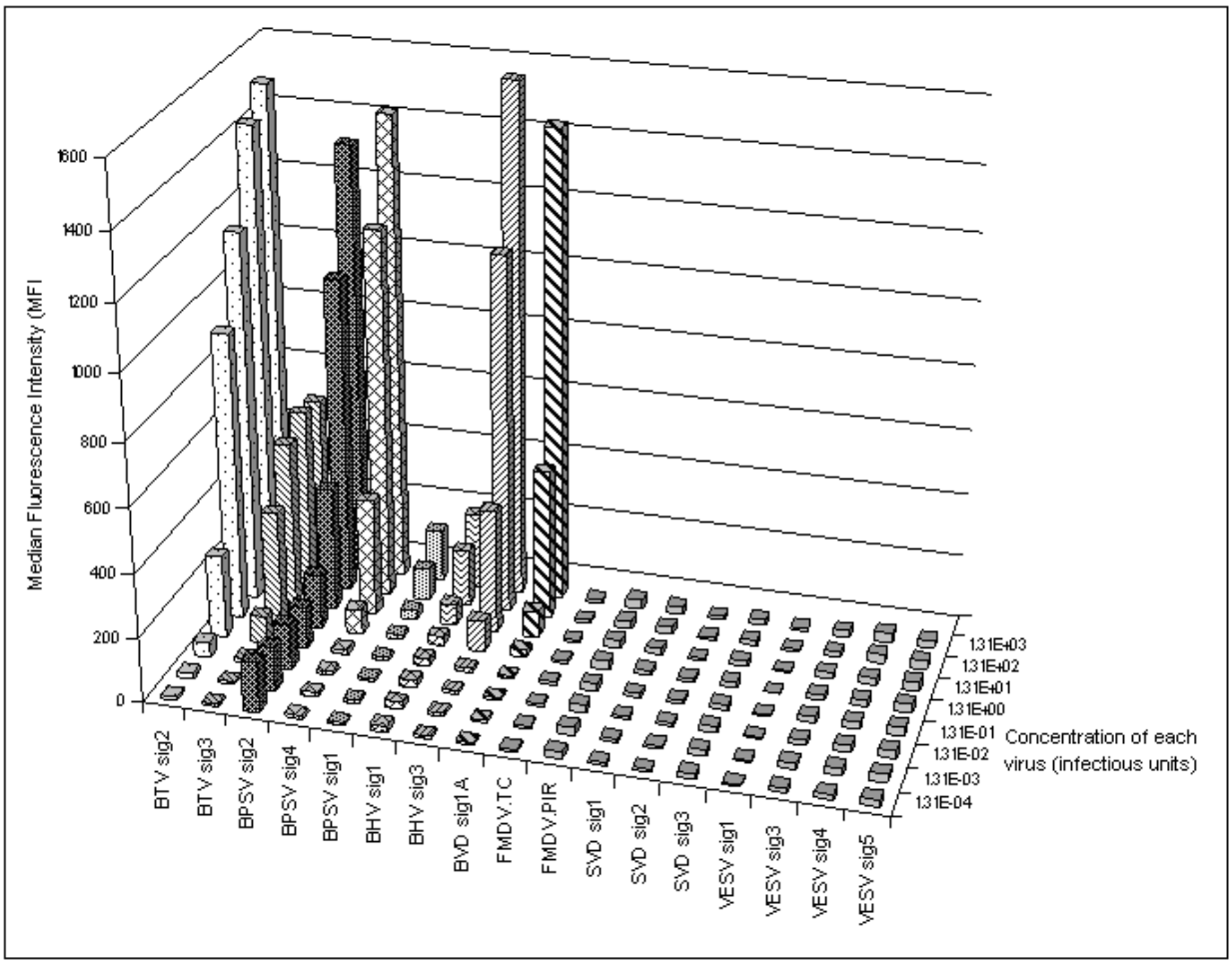

Figure 3. A specific and robust response of the multiplex assay to a mixture two different RNA and two DNA nucleic acids from four target viruses (BTV, BPSV, BHV and BVD). MFI of each assay is plotted on the z axis, viral concentration (shown as serial dilutions) along the y axis, and each individual signature is shown along the x axis. At the nucleic acid concentration of 1.3E -04 all the signatures show background MFI values. As the concentration of viral nucleic acid increases for the target viruses the MFI values for those assays are seen to tirate. The other channels for non target viruses remain unreactive even in the presence of this complex viral nucleic acid mixture.

Table 1. Summary of Components of Multiplexed Panel

Signature	Forward Primer (5' -3')	Reverse Primer (5' -3')	Probe (5' -3')	Amplicon length	Gene Target
Bovine Herpes Virus sig 1	GTGCCAGCCGCGTAAAAG	GACGACTCCGGGCTCTTTT	TCCTGGTTCCAGAGCGCTAACATGGAG	140bp	Glycoprotein C (UL43)
Bovine Herpes Virus sig 3	TGAGGCCTATGTATGGGCAGTT	GCGCGCCAAACATAAGTAAA	AAATAACACGGTGTGCACTTAAATAAGATTCGCG	114bp	Glycoprotein B (UL27)
Bovine Papular Stomatitis Virus 1	GCAGATGCGCTCCTGGTT	GCACCTCTGCTGCTGCAA	CCGACTCCGACGTGGAGAACGTG	178bp	DNA Packaging Protein/ATPase
Bovine Papular Stomatitis Virus 2	GATGGCCGTGCAGCTCTT	CGTACAAGATCACGGCCAACT	TGTACGGGCTCATGGGCTTCCG	95bp	DNA Polymerase
Bovine Papular Stomatitis Virus 4	GCAGCAGTGCACCACGTAGT	CGCTGAACCCGTACATCCT	GACTTCGAGGCGGACAACAAGCG	167bp	Early Transcription Factor [VETFL]
Foot and Mouth Virus (Tetracore)	ACTGGGTTTTACAAACCTGTGA	GCGAGTCCTGCCACGGA	GTCCCACGGCGTGCAAAGGA	107bp	3D Polymerase
Foot and Mouth Virus (Pirbright)	CACYTYAAGRTGACAYTGRTACTG GTAC	CAGATYCCRAGTGWCICITGTTA	CCTCGGGGTACCTGAAGGGCATCC	97bp	5' UTR
Bovine Viral Diarrhea sig 1	GGTAGTCGTCAGTGGTTCGAC	CATGTGCCATGTACAGCAGAGAT	CCTCGTCCACGTGGCATCTCGAG	195 bp	Mature Peptide N-Pro
Bluetongue Virus sig 2	GCACCCTATATGTTTCCAGACCA	CAGCTAACTCTTCAGCCACACG	CTAACTCGTGGGCCAATCATCATCTTCTGT	271bp	VP1 RNA polymerase
Bluetongue Virus sig 3	AGAATTCAGGATGGGCAGGA	GCACAATTCCCATCCCCTTA	CCATCACACCATTATACTGTACCCGCGTAGC	187bp	NS2 RNA binding protein
Swine Vesicular Disease 1	CAGGATAATTTCTTCCAAGGGC	ACGTGAACATTTCGAGCTTCC	TGCATTGTGTCTGATGGTACAACTTGTGACG	349bp	Viral Capsid Protein/VP1
Swine Vesicular Disease sig 2	GACTTGTTGTGGCTGGAGGA	CAGCGCCATGGTGAGGTAG	TGACCGTAATGAGGTCATCGTGATTTCTCAC	281bp	Membrane Permeability Enhancement [Protein 2B]
Swine Vesicular Disease sig 3	GACAAAGTGGCCAAGGGAAA	CACGTAAACCACACTGGGCT	CTGGCGTCATAGCCTGAATAGTCAAACGCTA	248bp	RNA Dependant RNA Polymerase [Protein3D]
VESV 1	GCCTTCTCCCTTCCCAAAA	TGAAGGAATGGTTCCGTCAGT	CATCATCGTTGATAACCTTAGATGTGCAATTTGG	153bp	Putative N-terminal Leader Protein [2B Ortholog]
VESV 3	GGGAATGAGGTGTGCATCATT	CACGTCTTGATGTTGGCTTGAC	AAATTGGCATAATCAACCTTGTCAGATGAGTCG	199bp	NTPase Protein [2C Ortholog]
VESV 4	GGTCGCTCTCACTGATGATGAGTA	GGTGTTATCAGCACCCATTGC	GCTCGGTGCCTGAGTTGGAGGAAG	124bp	Putative VPg
VESV 5	ACCACCTCTGGAAACATCTATGG	TTTGTGCACGTGTCACGAAT	CGGGACGGGCATTTGTCACCA	200bp	Putative Cysteine Protease
Maritima 7 (NC)	N/A	N/A	CAAAGTGGGAGACGTCGTTG	N/A	Assay Negative control
Biotin-maritima 7 (FC)	N/A	N/A	CAAAGTGGGAGACGTCGTTG	N/A	Stepavidin Phycoerythrin control
Cy3-labeled Maritima 7 (IC)	N/A	N/A	CAAAGTGGGAGACGTCGTTG	N/A	Instrument control
Alien-RNA PCR control	Propriatary sequence	Propriatary sequence	Propriatary sequence	N/A	PCR and extraction control

Table 2. Summary of Assay Development, Optimization and Characterization

Target analyte	#of computer-generated candidate signatures	# of signatures forwarded to Real-time PCR	# of signatures released to multiplex	# of signatures in final panel	# target strains tested singleplex	# target strains tested multiplex	# near-neighbor strains tested
BPSV	8	7	4	3	4	2	9
Orf	8	7	4	3	3	1	9
BHV	177	101	4	2	10	13	20
BVD	1	1	1	1	1	2	0
FMDV	4	4	4	2	29	7	42
BTV	8	8	4	2	5	5	4
SVD	4	4	4	3	30	11	47
VESV	44	20	6	4	12	11	2

Table 3. Summary of Multiplexed Assay Performance Against Selected Strains

Signature	Test Strain	Multiplex LOD [Infectious Units]	Background MFI	Threshold MFI
Bovine Herpes Virus-1 #1	CO Vaccine	500	30	>49
Bovine Herpes Virus-1 #3	CO Vaccine	500	40	>43
Bovine Papular Stomatitis Virus #1	Texas A&M	500	30	>35
Bovine Papular Stomatitis Virus #2	Texas A&M	50	65	>400
Bovine Papular Stomatitis Virus #4	Texas A&M	500	35	>41
Foot and Mouth Disease Virus (Tetracore)	01 Korea	$2.7X10^{-1}$	10	>42
Foot and Mouth Disease Virus (Pirbright)	01 Korea	$2.7X10^{2}$	10	>60
Bovine Viral Diarrhea #1	Singer Cytopathic	50	30	>40
Bluetongue Virus #2	NVSL serotype 13	5	30	>55
Bluetongue Virus #3	NVSL serotype 13	50	20	>31
Swine Vesicular Disease #1	ITL 1-66	20	8	>38
Swine Vesicular Disease #2	ITL 1-66	200	10	>28
Swine Vesicular Disease #3	ITL 1-66	20	16	>40
VESV #1	E54/A48	1.3	5	>24
VESV #2	A48	$1.3X10^{-3}$	18	>39
VESV #4	E54/A48	$1.3X10^{-2}$	17	>105
VESV #5	A48	$1.3X10^{-3}$	20	>56

Notes: Data is reported in absolute units, assuming that 5 µl of sample is added to each reaction. Two methods were used for determination of infectious units: FMDV strains were titered in plaque-forming units. All other viruses were quantitated in tissue culture infectious doses 50% endpoint (TCID50). Infectious units refer to either TCID50 or PFU, and are not extrapolations from an equation.